Loss of Scribble causes cell competition in mammalian cells

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Summary
In Drosophila, normal and transformed cells compete with each other for survival in a process called cell competition. However, it is not known whether comparable phenomena also occur in mammals. Scribble is a tumor suppressor protein in Drosophila and mammals. In this study we examine the interface between normal and Scribble-knockdown epithelial cells using Madin–Darby Canine Kidney (MDCK) cells expressing Scribble short hairpin RNA (shRNA) in a tetracycline-inducible manner. We observe that Scribble-knockdown cells undergo apoptosis and are apically extruded from the epithelium when surrounded by normal cells. Apoptosis does not occur when Scribble-knockdown cells are cultured alone, suggesting that the presence of surrounding normal cells induces the cell death. We also show that death of Scribble-knockdown cells occurs independently of apical extrusion. Finally, we demonstrate that apoptosis of Scribble-knockdown cells depends on activation of p38 mitogen-activated protein kinase (MAPK). This is the first demonstration that an oncogenic transformation within an epithelium induces cell competition in a mammalian cell culture system.

Key words: MDCK cells, Scribble, Cell competition, p38 MAPK

Introduction
Since the first oncogene Src was identified, a variety of oncoproteins and tumor suppressor genes have been found, and cellular functions and downstream signaling pathways of the encoded proteins have been revealed (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Most of these studies, however, the fact that transformation occurs in a single normal cell and that the transformed cell grows while being surrounded by neighboring normal cells has been largely overlooked. Thus, it is still not clearly understood what happens at the interface between normal and transformed cells at the initial stage of carcinogenesis. In Drosophila melanogaster, various phenomena have been reported to occur at the interface between normal and transformed epithelial cells. In particular, normal and transformed cells often compete with each other for survival. For example, when Drosophila Myc-overexpressing cells contact wild-type cells, wild-type cells undergo apoptosis and Myc-overexpressing cells proliferate and fill the vacant spaces (de la Cova et al., 2004; Moreno and Basler, 2004). By contrast, when Lethal giant larvae (Lgl)-knockout cells are surrounded by wild-type cells, Lgl-knockout cells die by apoptosis (Grzeschik et al., 2007; Tamori et al., 2010). These phenomena are called ‘cell competition’, which has been intensively studied in Drosophila (Baker and Li, 2008; Diaz and Moreno, 2005; Johnston, 2009).

However, it remains unknown whether comparable phenomena also occur in vertebrates (Fujita, 2011; Hogan et al., 2011). Scribble is a neoplastic tumor suppressor gene that was identified in Drosophila. In epithelia of Scribble homozygous mutant larvae, apicobasal cell polarity and proliferative control are lost, leading to multilayered amorphous tumor formation (Bilder and Perrimon, 2000). Scribble is a LAP (leucine-rich repeats and PDZ) protein that contains 16 leucine-rich repeat (LRR) and four PDZ [PSD95, Discs large and Zonula adherens-1 (ZO-1)] domains (Bilder and Perrimon, 2000) and is localized at the basolateral membrane in Drosophila and mammalian epithelial cells. Scribble has also been shown to function as a tumor suppressor protein in mice (Zhan et al., 2008), and decreased Scribble expression is observed in human colon and breast cancers (Gardiol et al., 2006; Navarro et al., 2005). In addition, Scribble has been reported to be involved in cell competition in Drosophila (Brumby and Richardson, 2003). When clones of homozygous scrib mutant cells are surrounded by wild-type cells in eye imaginal discs, scrib mutant cells are eliminated from the epithelium by Jun N-terminal kinase (JNK) pathway-mediated apoptosis. By contrast, when all epithelial cells are scrib mutant cells, they do not die, but overproliferate and form tumors. These data suggest that the presence of surrounding wild-type cells induces apoptosis of scrib mutant
cells. The underlying molecular mechanism is not fully understood, although the involvement of endocytic activation of Eiger/TNF and induction of phagocytosis has been suggested (Igaki et al., 2009; Ohsawa et al., 2011). In this study, we show that loss of Scribble causes cell competition in mammalian cells and investigate the molecular mechanism whereby death of Scribble-knockdown cells is induced.

**Results**

**Effect of Scribble knockdown on cell polarity and morphology in MDCK cells**

To examine the interaction between normal and Scribble-knockdown epithelial cells, we established MDCK epithelial cells stably expressing Scribble shRNA in a tetracycline-inducible manner (MDCK-pTR Scribble shRNA cells). At 48 hours after tetracycline addition, the expression level of Scribble was knocked down by 90% (Fig. 1A). Expression of other intercellular junction proteins, including E-cadherin and β-catenin, was not affected (Fig. 1B). Genetic studies in *Drosophila* have revealed that three tumor suppressor proteins, Scribble, Discs large (Dlg), and Lethal giant larvae (Lgl), cooperatively regulate cell polarity (Bilder et al., 2000). However, expression of neither Lgl nor Dlg was affected by knockdown of Scribble (supplementary material Fig. S1). As previously reported (Qin et al., 2005), Scribble-knockdown MDCK cells lost epithelial morphology with a flattened appearance when cultured at low density (Fig. 1C). However, when cultured at high density, they maintained apicobasal polarity, at least to a certain extent, as shown by localization of gp135 at the apical domain and of ZO-1 at tight junctions (Fig. 1D; and data not shown). By contrast, the distribution of E-cadherin was significantly disrupted in Scribble-knockdown cells; there was some E-cadherin localized at cell–cell contact sites, but the majority of E-cadherin was localized at the basal membrane (Fig. 1D), which is comparable with observations in a previous report (Qin et al., 2005). In all subsequent experiments, we cultured cells at high density.

**Scribble-knockdown cells are removed from a monolayer of normal epithelial cells**

To examine the fate of Scribble-knockdown cells within a monolayer of normal cells, we labeled MDCK-pTR Scribble shRNA cells with a red fluorescent dye (CMTPX) and mixed them with normal MDCK cells at a ratio of 1:10. The mixture of cells was cultured in the absence of tetracycline until a monolayer was formed. We then added tetracycline to induce knockdown of Scribble and followed the fate of Scribble-knockdown cells using time-lapse microscopy. To identify dead cells, we used SYTOX Blue fluorescent dye, which only passes through damaged cell membranes, binds DNA and emits blue fluorescence under violet light. After 60 hours of incubation with tetracycline, approximately 45% of Scribble-knockdown cells died and left the epithelial monolayer (Fig. 2A,B,E; supplementary material Movies 1, 2). Death of Scribble-knockdown cells was observed when either a small (Fig. 2A) or a large cluster (Fig. 2B) of Scribble-knockdown cells was surrounded by normal cells. Expression of exogenous human Scribble in Scribble shRNA cells significantly reduced cell death (supplementary material Fig. S2A,B), indicating that cell death is indeed caused by Scribble knockdown. Scribble shRNA cells did not die in the absence of tetracycline (Fig. 2C,E; supplementary material Movie 3). Importantly, fluorescently labeled Scribble-knockdown cells did not die when surrounded by unlabeled Scribble-knockdown cells (Fig. 2D,E). Collectively, these data suggest that the presence of surrounding normal cells induces death of Scribble-knockdown cells. This is the first demonstration that knockdown of a tumor suppressor protein causes cell competition in mammalian epithelial cells.

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**Fig. 1. Characterization of MDCK cells stably expressing Scribble shRNA in a tetracycline-inducible manner.**

(A,B) Effect of tetracycline addition on the expression of Scribble (A), E-cadherin and β-catenin (B) in MDCK-pTR Scribble shRNA cells. MDCK or MDCK-pTR Scribble shRNA cells were incubated with tetracycline for the indicated times, and cell lysates were examined by western blotting using anti-Scribble, anti-E-cadherin, anti-β-catenin or anti-GAPDH antibody. (C) Phase-contrast images of MDCK-pTR Scribble shRNA cells cultured in the presence of tetracycline for the indicated times. (D) Effect of Scribble knockdown on cell polarity. MDCK-pTR Scribble shRNA cells were incubated without or with tetracycline for 72 hours and were analyzed by immunofluorescence using anti-gp135 and anti-E-cadherin antibodies. Nuclei were stained with Hoechst 33342 dye. Scale bars: 15 μm (C), 10 μm (D).
Scribble-knockdown cells undergo apoptosis independently of apical extrusion

Next, we examined the cell death of Scribble-knockdown cells by immunofluorescence. We found that apically extruded Scribble-knockdown cells were always positively immunostained with anti-active caspase-3 antibody and had fragmented nuclei, indicating that they had undergone apoptosis (Fig. 3A). By contrast, when cells expressing scramble shRNA were surrounded by normal cells, no significant active caspase-3 staining was observed (supplementary material Fig. S2C,D). 48 hours after tetracycline addition, active caspase-3-positive Scribble-knockdown cells often remained within the epithelial monolayer (supplementary material Fig. S3A), suggesting that caspase activation might occur before apical extrusion. Bcl-2 homologous antagonist/killer (Bak) and Bcl-2 associated X protein (Bax) are proapoptotic members of the Bcl-2 family and promote apoptosis through their effect on mitochondria (Youle and Strasser, 2008). We found that Bak and Bax were activated in apically extruded Scribble-knockdown cells (Fig. 3B,C), suggesting the involvement of the mitochondrial apoptosis pathway in their death. Addition of the poly-caspase inhibitor Z-VAD-FMK blocked activation of caspase-3 (Fig. 3D; supplementary material Fig. S3B,C), but did not substantially affect activation of Bak and Bax (Fig. 3D; supplementary material Fig. S3C; and data not shown) in Scribble-knockdown cells surrounded by normal cells, suggesting that Z-VAD-FMK-sensitive caspases are not required for activation of Bak and Bax. Surprisingly, addition of Z-VAD-FMK did not significantly block death of Scribble-knockdown cells surrounded by normal cells (supplementary material Fig. S3D). It is possible that Z-VAD-FMK-insensitive caspases (Chauvier et al., 2007) are involved in the cell death or that inhibition of caspases induces alternative cell death pathways.

To determine whether Scribble-knockdown cells surrounded by normal cells die before or after apical extrusion, we treated cells with the myosin-II inhibitor blebbistatin, which blocks apical extrusion of apoptotic cells from the epithelial monolayer (Rosenblatt et al., 2001). In the presence of blebbistatin, the extrusion of apoptotic Scribble-knockdown cells, as denoted by fragmented nuclei and positive staining for the active form of caspase-3, was inhibited, and cells remained within the monolayer (Fig. 3E). Under these conditions, 24% of Scribble-knockdown cells surrounded by normal cells were positive for active caspase-3 (Fig. 3E,F). By contrast, when Scribble shRNA cells were cultured with normal cells without tetracycline, or Scribble-knockdown cells were cultured alone, positive immunostaining for active caspase-3 was not frequently observed (Fig. 3F). Addition of tetracycline did not affect activation of caspase-3 in normal cells or scramble-knockdown cells (supplementary material Fig. S3E). These data suggest that activation of the apoptotic pathway of Scribble-knockdown cells surrounded by normal cells can occur independently of apical extrusion.

Molecular mechanisms of death of Scribble-knockdown cells

It has been reported in Drosophila that JNK plays an important role in death of outcompeted cells in cell competition (Brumby and Richardson, 2003; Igaki et al., 2009; Moreno and Basler, 2004; Tamori et al., 2010). Therefore, we next examined the role of JNK in the death of Scribble-knockdown MDCK cells. We did not observe phosphorylation of JNK in Scribble-knockdown cells...
surrounded by normal cells (data not shown). In addition, the JNK-specific inhibitor SP600125 did not significantly inhibit death of Scribble-knockdown cells surrounded by normal cells (supplementary material Fig. S4A). p38 MAPK (MAPK14) is another MAPK that is activated by various types of cellular stresses and is involved in apoptosis (Zarubin and Han, 2005). Immunofluorescence analyses with an antibody against phosphorylated p38 MAPK demonstrated that protein activity was significantly enhanced in Scribble-knockdown cells surrounded by normal cells, but not in normal cells or Scribble-knockdown cells cultured alone (Fig. 4A,B; supplementary material Fig. S5). Addition of the p38 MAPK inhibitor SB202190 significantly reduced activation of caspase-3 and death of Scribble-knockdown cells surrounded by normal cells (Fig. 5A; supplementary material Fig. S4B). Furthermore, expression of a dominant-negative form of p38 MAPK strongly suppressed death of Scribble-knockdown cells surrounded by normal cells (Fig. 5B,C). Collectively, these data indicate that p38 MAPK, not JNK, plays a crucial role in cell competition between normal and Scribble-knockdown MDCK cells. Finally, inhibition of p38 MAPK significantly suppressed activation of Bak in Scribble-knockdown cells surrounded by normal cells (Fig. 5D), suggesting that the mitochondrial apoptosis pathway is one of the downstream targets of p38 MAPK.
Discussion

In this study, we have shown that Scribble-knockdown MDCK cells undergo apoptosis when surrounded by normal MDCK cells. It has been reported that apical extrusion of apoptotic epithelial cells occurs in a myosin-II-dependent manner at an early stage of apoptosis (Rosenblatt et al., 2001). Indeed, we have observed that caspase-3-positive Scribble-knockdown cells are apically extruded from the monolayer of normal cells. Inhibition of myosin-II with blebbistatin inhibits apical extrusion, but not cell death of Scribble-knockdown cells. These results indicate that apoptosis of Scribble-knockdown cells occurs prior to extrusion and is not caused by anoikis, which is compatible with observations for cell competition in Drosophila (Moreno and Basler, 2004; Tamori et al., 2010). In Drosophila, apoptotic cells are basally extruded and easily detected by immunofluorescence. By contrast, extruded scribble-knockdown MDCK cells loosely attach to the apical surface of the epithelium and are frequently lost during the washing steps of immunofluorescence. Thus time-lapse analyses are indispensable to properly quantify apical extrusion of apoptotic transformed cells in mammalian epithelial cell culture systems.

The molecular mechanism whereby outcompeted cells undergo apoptosis in cell competition is largely unknown in Drosophila or mammals. It was previously believed that fast-proliferating cells outcompete slow-proliferating cells. However, several reports have revealed that differences in proliferation rates are not always involved in cell competition (Tamori et al., 2010). Indeed, there were no significant differences in proliferation rates between normal and Scribble-knockdown cells (supplementary material Fig. S6), suggesting that differences in proliferation rates may not play a vital role in the cell competition. It has been also proposed that different types of cells compete for soluble survival factors; this is called the ligand capture hypothesis. However, it is unlikely that death of Scribble-knockdown MDCK cells is caused by this mechanism because the frequency of cell death was not substantially affected by repeated replacement of culture medium. In Drosophila, the JNK pathway has been shown to be involved in cell competition (Brumby and Richardson, 2003; Moreno and Basler, 2004; Moreno et al., 2002; Tamori et al., 2010). Brumby and colleagues demonstrated that expression of dominant-negative JNK suppresses apoptosis of Scribble mutant cells surrounded by wild-type cells in the eye disc epithelium (Brumby and Richardson, 2003). However, our data indicate the involvement of p38 MAPK, not JNK, in cell competition between normal and scribble-knockdown MDCK cells. In addition, unlike studies in Drosophila (Igaki et al., 2009), endocytosis was not significantly increased in Scribble-knockdown cells, compared with surrounding normal MDCK cells (supplementary material Fig. S7). Thus, cell competition caused by loss of Scribble is, at least partially, mediated by different molecular mechanisms in Drosophila and mammals. Previously we demonstrated that JNK is involved in apoptosis of Mahjong-knockdown MDCK cells surrounded by normal MDCK cells (Tamori et al., 2010), suggesting the involvement of distinct signaling pathways in these two types of mammalian cell competition. ASK1 is one of the upstream activators of p38 MAPK, but we did not observe increased activation of ASK1 in Scribble-knockdown cells surrounded by normal cells (data not shown). How the presence of surrounding normal cells influences signaling pathways including p38 MAPK in Scribble-knockdown cells remains to be resolved. Recent studies revealed the involvement of two molecules, Flower and Sparc, in cell competition in Drosophila (Portela et al., 2010; Rhiner et al., 2010). It also remains to be clarified whether homologous proteins also play a role in cell competition in mammals.

Another important question is whether a comparable cell competition phenotype can be observed in vivo in mammals. In most of the conventional mouse carcinogenesis model systems, epithelial-tissue specific promoters have been used where genetic changes are induced within the entire epithelium. These are useful systems to study cell-autonomous signaling pathways, but are not suitable to examine the interface between normal and transformed epithelial cells. Therefore, a novel murine model...
system needs to be established where oncogenic alterations can be induced in a mosaic manner within epithelial tissues in combination with in vivo live cell imaging.

Cell competition is a newly emerging field in cancer biology, which sheds light on the interaction between normal and transformed epithelial cells at the early stage of carcinogenesis. It is hoped that future studies will lead to a novel type of cancer prevention and treatment.

Materials and Methods
Plasmids, antibodies and materials
To construct pSUPERIOR Scribble shRNA, Scribble shRNA oligonucleotides (5'-GATCCCCAGATGTTCTCCAGCAAGTTCAAGAGAATGCTGAGA-3' and 5'-TCGAGAAACACATGTCGATGATCGCAAGTTCCTCT-3') (Qin et al., 2005) were cloned into Bgl II and XhoI sites of pSUPERIOR-neo+gfp (Oligogene). To construct pSUPERIOR scramble shRNA, scramble shRNA oligonucleotides (5'-GATCCCCAGCCGCTATGCTGAGAATGCTGAGA-3' and 5'-TCGAGAAACACATGTCGATGATCGCAAGTTCCTCT-3') and 5'-TCGAGAAACACATGTCGATGATCGCAAGTTCCTCT-3') were cloned into Bgl II and XhoI sites of pSUPERIOR-neo+gfp. pcDNA4/TO/human Scribble was constructed by digestion of pEGFP-C1 human Scribble (kindly provided by Jean-Paul Borg, The INSERM Cancer Research Centre, Marseille, France) with Bam HI and XhoI and ligation into Bam HI and XhoI sites of pcDNA4/TO (Dupre-Crochet et al., 2007). pcDNA4/TO-GFP-p38DN was constructed by amplification of p38αK33N from pcDNA3-HA-p38α and ligation into XhoI and Pst I sites of pcDNA4/TO-GFP. pcDNA3-HA-p38α was a kind gift from Mutsuhiro Takegawa, Nagoya University, Nagoya, Japan).

Rabbit antibodies against phosphorylated p38 MAPK (T180/Y182), mouse anti-phosphorylated p38 MAPK (T180/Y182), mouse anti-active-Bak antibody and mouse anti-tubulin antibody were obtained from Santa-Cruz Biotechnology. Goat anti-Scribble and mouse anti-Dlg1 (2D11) antibodies were obtained from Cell Signaling Technology. Goat anti-Scribble and mouse anti-Dlg1 (2D11) antibodies were obtained from Cell Signaling Technology. Goat anti-Scribble and mouse anti-Dlg1 (2D11) antibodies were obtained from Cell Signaling Technology. Goat anti-Scribble and mouse anti-Dlg1 (2D11) antibodies were obtained from Cell Signaling Technology.

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dilution of 1:50, and all other primary antibodies were used at a dilution of 1:100. Alexa Fluor secondary antibodies (Invitrogen) were used at a dilution of 1:200. Alexa Fluor-647-conjugated phallolidin (Invitrogen) was used at 1.5 μg/ml. For western blotting, anti-GAPDH antibody was used at 1:5000, and all other antibodies at 1:1000. Hoechst 33342 (Invitrogen) was used to visualize nuclei. Cells were fluorescently labeled with a green CMFDA or red CMTX dye (Invitrogen) according to manufacturer’s instructions. Inhibitors were used at the following concentrations: JNK1,2,3 inhibitor SP600125 (Sigma-Aldrich), 5 μM; p38MAPK inhibitor SB202190 (Calbiochem), 10 μM; myosin II inhibitor (S)-(-) blebbistatin (Toronto Research Chemicals), 30 μM; caspase inhibitor Z-VAD-FMK (Calbiochem), 100 μM. For immunofluorescence analyses, inhibitors were added 24 hours before fixation. For live-image analyses, inhibitors were added immediately before analyses and incubated for 40 hours.

Endocytosis assay
Cells were seeded onto glass coverslips in a 12-well plate at a density of 8 × 10^4 cells per well. For mixed cultures, 7.2 × 10^4 unstained cells were mixed with 0.8 × 10^5 stained cells. Tetracycline was added 6 hours after seeding. 64 hours after addition of tetracycline, Rhodamine-conjugated dextran (10,000 kDa) (Invitrogen) was added at a final concentration of 400 μg ml^-1, and cells were further incubated for 30 minutes at 37°C. Cells on coverslips were washed once with ice-cold PBS, fixed in ice-cold 4% paraformaldehyde (PFA) for 15 minutes, and mounted with Mowiol.

Imaging
Phase-contrast images were acquired at ambient temperature using a Leica DMiRB microscope with a 20 × 0.40 NA objective lens (Leica) and a Hamamatsu C4742-95 Orca camera. Images were acquired using Openlab software (PerkinElmer). Confocal images were acquired using a Leica TCS SPE confocal microscope with an 63 × 1.30 oil-immersion objective lens and Leica Application Suite (LAS) (Leica). Images were colored, and contrast and brightness were enhanced linearly using Photoshop CS4 (Adobe).

Data analyses
Student’s t-tests were used to determine P values because this test requires variables with no fixed limits. For Fig. 4B, using Metamorph 6.0 digital analysis software (Universal Imaging), p38 MAPK fluorescence intensity was measured at eight random points (50 pixels each) in each cell, and the mean of the values was used for statistical analyses. For supplementary material Fig. S7, confocal images of 7–9 z sections at 0.42 μm intervals were acquired and processed using a maximum projection tool with Las AF software (Leica). The total number of fluorescent red puncta within each cell was counted, and the mean of the values was used for statistical analyses.

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Fig. S1

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Fig. S2

(A) Western blot analysis of Scribble and tubulin expression in MDCK cells with different treatments: Scribble KD, Scribble rescue, and scrambled shRNA.

(B) Graph showing the percentage of dead cells relative to total stained cells. The Scribble KD:MDCK Rescue:MDCK ratio is indicated.

(C) Western blot analysis of Scribble and GAPDH expression in MDCK cells with different treatments: pTR scramble shRNA and Tetracycline concentration.

(D) Graph showing the percentage of active caspase-3 positive cells relative to total stained cells. The pTR scramble shRNA:MDCK 1:10 ratio is indicated.

Fig. S2
Fig. S3
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A

Fig. S4

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dead/total stained cells (percentage)

[Bar chart with two groups: - and + SP600125, showing data points with error bars.]

B

Cas-3-positive cells (percentage)

[Bar chart with three groups: - Tetracycline, + Tetracycline, + SB202190, showing data points with error bars.]

* *
Fig. S6
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Normal MDCK

**Fig. S7**

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**A**

- CMFDA
- Dextran

**B**

- **Number of dextran-labeled puncta/cell**
  - Normal MDCK
  - Scribble

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Scribble shRNA: normal MDCK 1:10